

AD_____

Award Number: DAMD17-02-1-0389

TITLE: CRE Activation in Antiestrogen Resistance

PRINCIPAL INVESTIGATOR: Bianca P. Gomez
Robert Clarke, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20007

REPORT DATE: May 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20041118 093

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE May 2004		3. REPORT TYPE AND DATES COVERED Annual Summary (1 May 2003 - 30 Apr 2004)
4. TITLE AND SUBTITLE CRE Activation in Antiestrogen Resistance			5. FUNDING NUMBERS DAMD17-02-1-0389	
6. AUTHOR(S) Bianca P. Gomez Robert Clarke, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Georgetown University Washington, DC 20007 E-Mail: BPG5@georgetown.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) HXBP-1, a basic leucine zipper protein (bZIP), is transcription factor that belongs to the activating transcription factor/ cAMP response element-binding protein (ATF/CRE) family of transcription factors and can bind to and activate the cyclic AMP response elements (CREs). In our previous studies, serial analysis of gene expression has shown that hXBP-1 may contribute to antiestrogen resistance in human breast cancer cells. In antiestrogen resistant cells CRE-regulated transcription (promoter-reporter assay) activity is increased 4-fold. Thus, the purpose of our experiments is to determine whether hXBP-1/CRE contribute to antiestrogen resistance by over- expressing hXBP-1 in cell lines that are sensitive to antiestrogen. MCF7 cells were transfected with hXBP-1 cDNA. Western blotting showed increased expression in transfectants; activity of this protein was confirmed in a promoter – reporter assay measuring luciferase activity as an indicator of CRE activation. Preliminary data show that MCF7 cells over-expressing hXBP-1, when exposed to antiestrogens such as 4-hydroxy- Tamoxifen and ICI 182, 780, are able to survive the growth inhibitory effects of these antiestrogens when compared to the parental MCF7 cells. Similar observations were observed when both cell types were grown in media devoid of estrogen (E2).				
14. SUBJECT TERMS HXBP-1- Human X-box binding protein, CRE- cyclic AMP response elements, E2-estrogen				15. NUMBER OF PAGES 13
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

N/A Where copyrighted material is quoted, permission has been obtained to use such material.

N/A Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

N/A Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

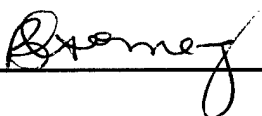
In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



Date

PI - Signature

Table of Contents

Cover	1
SF 298	2
Foreword	3
Table of Contents	4
Introduction	5
Body	6-9
Key Research Accomplishments	10
Reportable Outcomes	11
Conclusions	12
References	13

INTRODUCTION

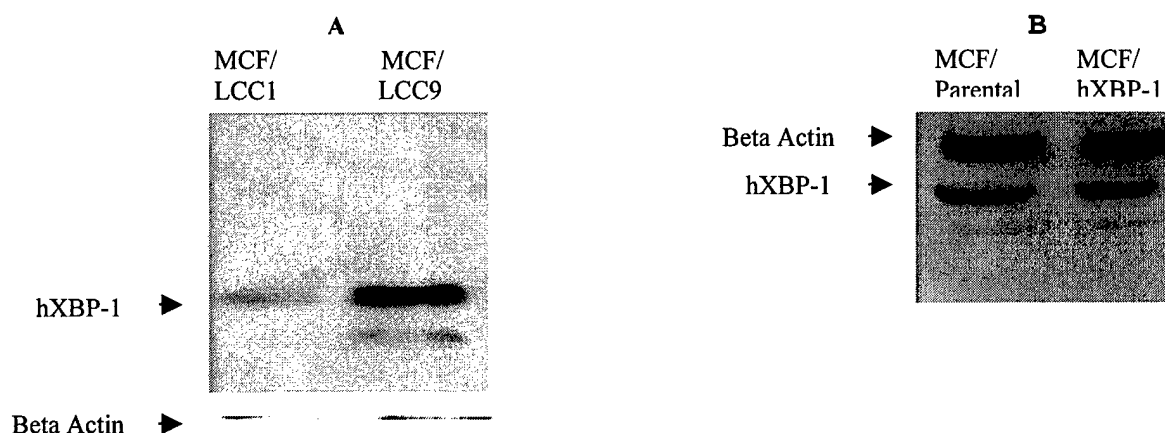
The rationale for these experiments is based on the fact that most patients with initial responsive breast tumors to antiestrogens acquire antiestrogen resistance (1). Although several possible mechanisms such as mutations in the estrogen receptor (ER) and loss of the ER expression have been associated with antiestrogen resistance, other possible mechanisms that can be considered to contribute to antiestrogen resistance may also include Tamoxifen stimulated resistance mechanisms (1). Tamoxifen (TAM) or ICI 46, 477 is the most widely used antiestrogen for breast cancer treatment. Faslodex (ICI 182, 780) is another antiestrogen therapy used for breast cancer treatment (2). It is effective in patients who have acquired resistance to TAM treatment. In these experiments MCF7 cells stably transfected with pcDNA 3.1/hXBP-1 will be used to determine resistance to these antiestrogens when hXBP-1 is over-expressed.

We hypothesize that the over expression of hXBP-1 in cell lines that are sensitive to antiestrogens will confer estrogen independence and antiestrogen resistance. Following stable transfection with the pcDNA/hXBP-1 construct in MCF7 breast cancer cells that are sensitive to antiestrogens such as 4-OH Tamoxifen (TAM) or Faslodex (ICI, 182,780), these cells appear to have a better survival rate when compared to the non-transfected MCF7 cells thereby conferring resistance to these antiestrogens. This resistance to antiestrogens may occur by growth induction or by apoptosis blockage. The up-regulation of hXBP-1 has shown the expected increased CRE transcriptional activation, which was measured by promoter- reporter assays.

BODY

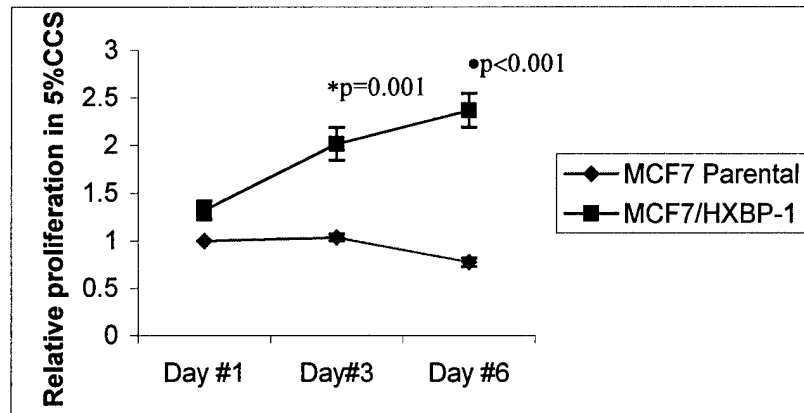
Task 1. To determine if sensitive cells can become resistant to antiestrogens Tamoxifen and Faslodex by the over-expression of hXBP-1 in breast cancer cells (Months 1-24)

Figure 1. The differential levels of hXBP-1 expression in MCF7 cells. A, basal expression levels. B, over expression after stable transfection.



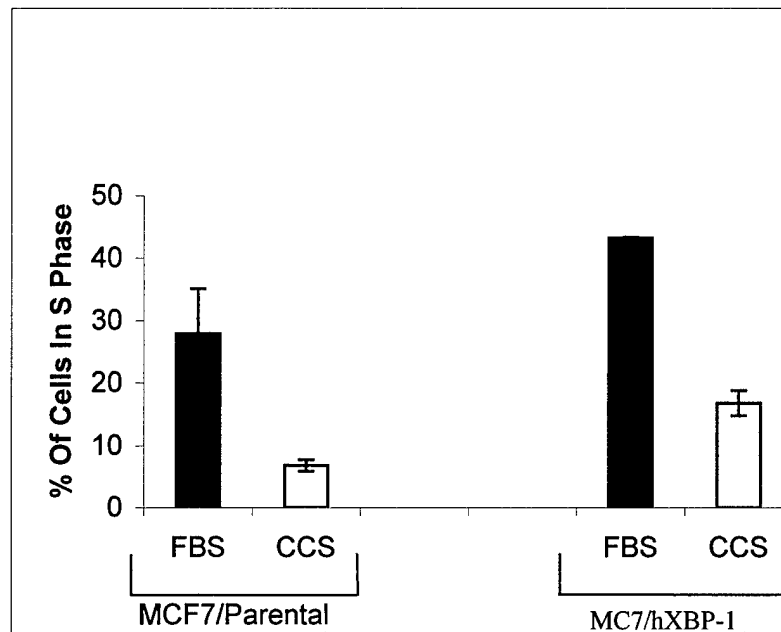
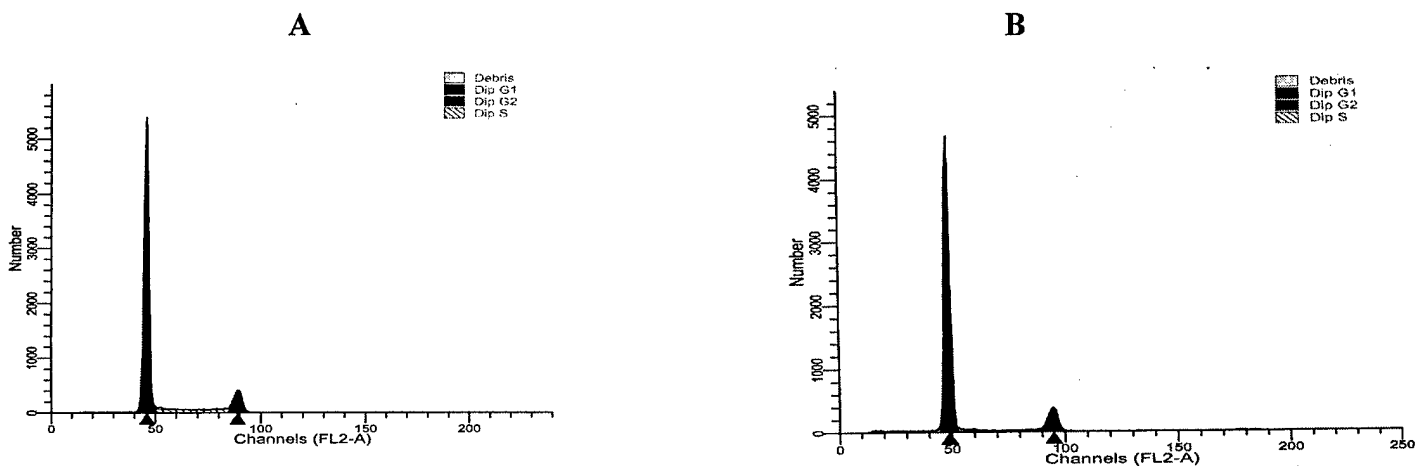
For western blot analysis 60ug of total protein was isolated from hXBP-1 transfected MCF7 cells and resolved by electrophoresis using NuPage 10% Bis-Tris gels and blotted onto nitrocellulose membranes. After blocking, the nitrocellulose membrane was washed and incubated with rabbit polyclonal hXBP-1 primary antibody (1:100) (Santa Cruz). Beta actin primary antibody (1:5000) was used to compare loading. Antigen-antibody complexes were then visualized with the ECL detection system (Amersham) and exposed to film.

BODY CONT'D

Figure 2. Growth efficiency in the absence of estrogens.

MCF7 cells over expressing hXBP-1 and parental MCF7 cells were allowed to grow in IMEM without phenol red supplemented with 5% charcoal stripped calf serum (CCS) for 72 hours. After growth devoid of estrogen, the cells were plated at a density of 3.5×10^4 cells/well into 12 well plastic tissue culture plates and allowed to grow. On days 1, 3 and 6 post plating the cells were then trypsinized, resuspended in 1X PBS and counted using a Beckman coulter counter (Beckman Coulter Corp., Fullerton, CA) to determine growth efficiency. P-values were obtained using the Student-Newman-Keuls Method .

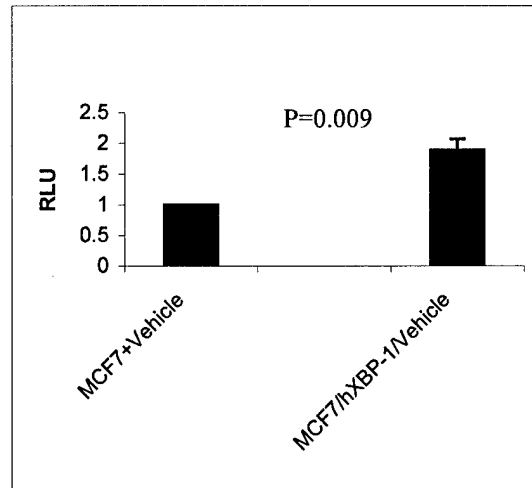
BODY CONT'D

Figure 3. Comparison of Cell Survival in 5% FBS and 5% CCS**Figure 4.** Alterations in cell cycle in 5% CCS media. A, MCF/7 hXBP-1 cells. B, MCF7/parental cells.

Cells stably transfected with pCDNA3.1/hXBP-1 or parental MCF7 cells were plated in T-75 cm² plastic tissue culture flasks at a density within 2×10^5 – 1×10^6 and allowed to grow in 5% CCS or 5 % FBS for three days. Cells were then harvested and analyzed for alterations in cell cycle according to the method of Vindelov.

BODY CONT'D

Figure 5. Fold transcriptional activity of CRE in MCF7/Parental and MCF7/hXBP-1 cells.



3.5×10^4 cells/well were plated in 12 well plates and allowed to grow for 24 hours prior transfection. Cells were cotransfected with 0.4 μ g of CRE- luciferase plasmid. To account for possible differences in transfection efficiency, 0.1 μ g of a plasmid containing the *Renilla* luciferase gene was also cotransfected into the cells. Both plasmid DNAs were added to a mix of Eugene 6 (Roche) and serum free IMEM after their incubation at room temperature for 5 mins., after which an additional incubation of 15 mins. was carried out at room temperature. The transfection mix was added/well and incubated at 37°C for 3 hours. Cells were maintained in media containing serum. After transfection, fresh media containing 0.1% EtOH as vehicle was added to each well for 24 hours, cells were then lysed and the activation of the CRE-luciferase construct was measured using the Dual Luciferase Assay Kit (Promega). Luminescence was quantitated using a Lumat LB 9501 luminator (EG&G Berthold, Bundoora VIC, Australia). P-value was obtained using the Students t-test.

KEY RESEARCH ACCOMPLISHMENTS

The goal of task 1 was to show:

- Greater survival in media devoid of estrogens and antiestrogen containing media of MCF7 cells stably transfected with pCDNA 3.1 hXBP-1.

MCF7 cells over-expressing hXBP-1 have a significantly increased growth rate when compared to non-transfected MCF7 cells in media devoid of estrogen (5%CCS) through day #3 to #6 (**Fig. 2**; * $p=0.001$ and • $p<0.001$ respectively). **Figs. 3 and 4** also indicate that under estrogen deprived conditions the number of cells in S phase in cells over expressing hXBP-1 is approximately three times more than those in the non transfected cells. (P values are not indicated because of insufficient sampling; only two experiments were done so far). Furthermore, preliminary data show that MCF7 cells over-expressing hXBP-1, when exposed to antiestrogens such as 4-hydroxy Tamoxifen (TAM) and ICI 182,780, reduce sensitivity to these antiestrogens (growth curve data not shown). In addition, the same trend is evident in apoptosis assays using FITC annexin V/propidium iodide (data not shown).

- Measure CRE transcriptional activity to confirm hXBP-1 activity.

Fig. 5 indicates a significant ($p = 0.009$) two- fold increase in relative CRE activity in cells over- expressing hXBP-1 when compared to parental MCF7 cells.

REPORTABLE OUTCOMES

- ✓ Received a minority scholar travel award to the American Association for Cancer Research held in Orlando, March 27th-31st 2004.

The following abstract was presented

Human X-Box Binding Protein In Antiestrogen Resistance

Bianca Gomez, Rebecca Riggins, Yhuelin Zhu, Alan Zwart and Robert Clarke
Georgetown University, Lombardi Cancer Center, Washington D.C. 2007

HXBP-1 a basic leucine zipper protein (bZIP), is transcription factor that belongs to the activating transcription factor/ cAMP response element-binding protein (ATF/CRE) family of transcription factors and can bind to and activate the cyclic AMP response elements (CREs). In our previous studies, serial analysis of gene expression (SAGE) has shown that hXBP-1 may contribute to antiestrogen resistance in human breast cancer cells. In antiestrogen resistant cells, CRE-regulated transcription (promoter-reporter assay) activity is increased 4-fold. Thus, the purpose of our experiments is to determine whether hXBP-1/CRE contribute to antiestrogen resistance by over expressing hXBP-1 in cell lines that are sensitive to antiestrogen. MCF7 cells were transfected with hXB-1 cDNA. Western blotting showed increased expression in transfectants; activity of this protein was confirmed in a promoter – reporter assay measuring luciferase activity as an indicator of CRE activation. Preliminary data show that MCF7 cells over-expressing hXBP-1, when exposed to antiestrogens such as 4-hydroxy- Tamoxifen (TAM) and ICI 182, 780, are able to survive the growth inhibitory effects of these antiestrogens when compared to the parental MCF7 cells. Similar observations were observed when both cell types were grown in media void of estrogen. In vivo studies using ovariectomized nude mice cells will be inoculated with MCF7/hXBP-1 cells into opposite thoracic mammary glands as control and test cells to evaluate hormone sensitivity by the administration of estrogen, TAM or ICI will be in progress. These preliminary data suggest that hXBP-1 may be a factor associated with antiestrogen resistance in breast cancer. This work was supported by awards from the Department of Defense (DAMD 17-02-1-0389 and DAMD 17-02-1-0388)

CONCLUSIONS

1. Over-expression of hXBP-1 in MCF7 cells confers estrogen independent growth.
2. Preliminary data with ICI 182,780 (Faslodex) and 4-OH Tamoxifen (TAM) suggest that increased hXBP-1 expression reduces sensitivity to these antiestrogens.

Future goals:

To complete Task 1

- Perform more experiments to compare cell survival in 5%FBS and 5% CCS to determine any statistical differences between experimental groups.
- Perform further experiments to determine relative cell proliferation of MCF7/hXBP-1 and MCF7/parental cells in the presence of antiestrogens.
- Perform Real Time PCR and Western blot analysis to determine expression levels of IL6, BCL2, pS2, ER α and IRF1.
- Use mouse models to measure antiestrogen sensitivity.
- Screen clones of T47D cells that were transfected with pCDNA 3.1 hXBP-1 to determine hXBP-1 over-expression. Perform the above experiments as done in MCF7 cells.

Task 2

- Measure response to antiestrogens after transfection with CRE decoy oligonucleotides
- Select cells with low hXBP-1 activity
- Confirm hXBP-1 reduced activity by measuring CRE transcriptional activity
- Confirm cell sensitivity by measuring antiestrogen response.

REFERENCES

1. Clarke, R., Leonessa, F., Welch, J.N., and Skaar, T.C. Cellular and Molecular pharmacology of antiestrogen action and resistance. *Pharmacol Rev*, 53:25-71, 2001.
2. Howell, A., DeFriend, D., Robertson, J.F.R., Blamey, R.W., and Walton, P. Responses to a specific antiestrogen (ICI 182,780) in tamoxifen-resistant breast cancer. *Lancet*, 345: 29-30, 1995.